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A Novel Bioreactor for Stimulating Skeletal Muscle *In Vitro*

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For over 300 years, scientists have understood that stimulation, in the form of an electrical impulse, is required for normal muscle function. More recently, the role of specific parameters of the electrical impulse (i.e., the pulse amplitude, pulse width, and work-to-rest ratio) has become better appreciated. However, most existing bioreactor systems do not permit sufficient control over these parameters. Therefore, the aim of the current study was to engineer an inexpensive muscle electrical stimulation bioreactor to apply physiologically relevant electrical stimulation patterns to tissue-engineered muscles and monolayers in culture. A low-powered microcontroller and a DC–DC converter were used to power a pulse circuit that converted a 4.5 V input to outputs of up to 50 V, with pulse widths from 0.05 to 4 ms, and frequencies up to 100 Hz (with certain operational limitations). When two-dimensional cultures were stimulated at high frequencies (100 Hz), this resulted in an increase in the rate of protein synthesis (at 12 h, control [CTL] = 5.0 ± 0.16; 10 Hz = 5.0 ± 0.07; and 100 Hz = 5.5 ± 0.13 fmol/min/mg) showing that this was an anabolic signal. When three-dimensional engineered muscles were stimulated at 0.1 ms and one or two times rheobase, stimulation improved force production (CTL = 0.07 ± 0.009; 1.25 V/mm = 0.10 ± 0.011; 2.5 V/mm = 0.14146 ± 0.012; and 5 V/mm = 0.03756 ± 0.008 kN/mm^2) and excitability (CTL = 0.53 ± 0.022; 1.25 V/mm = 0.44 ± 0.025; 2.5 V/mm = 0.41 ± 0.012; and 5 V/mm = 0.60 ± 0.021 V/mm), suggesting enhanced maturation. Together, these data show that the physiology and function of muscles can be improved *in vitro* using a bioreactor that allows the control of pulse amplitude, pulse width, pulse frequency, and work-to-rest ratio.

Introduction

One of the most important cues for functional muscle development is the electrical impulse the tissue receives from the central nervous system via the motor neurons.[1] When muscles are denervated *in utero*, the aneural myotubes fail to develop fully.[1–4] Instead, the muscles are developmentally arrested at the primary myotube stage and do not continue to develop into adult phenotype myofibers. Similarly, all attempts to date to engineer muscle have resulted in a developmental block at the level of primary myotubes.[5] During development, the transition from primary myotubes to secondary myotubes is dependant on electrical activity. In the absence of electrical activity, secondary myotubes and adult muscle fibers fail to form.[4] Therefore, we hypothesized that if the electrical stimulation pattern can be reproduced *in vitro*, this may promote the transition toward adult myofibers within engineered muscles.

In denervated muscle *in vivo* and for *in vitro* tissue engineering experiments, the absence of the nerve requires that stimulation be provided by systems that emulate neural function using low-intensity electrical pulses. The use of electrical stimulation of muscle tissue has a long history[6]; however, control of important stimulation parameters such as pulse widths, frequency, and voltage amplitude has been poorly understood.[7] It is only within the last four decades that the effect of different stimulation patterns on muscle function has been fully appreciated.[8] One of the key aspects of muscle physiology that is determined by the parameters of stimulation is fiber type. In an elegant experiment, Salmons and Vrbova[9] demonstrated that the frequency of electrical input was a key determinant of whether a muscle was fast or slow. The importance of regulating the electrical impulse together with advanced electronic component technologies means that new stimulation systems (bioreactors) can be developed that enable more complex and physiologically relevant stimulation patterns to be applied. Many of these systems are developed in-house by individual laboratories to address specific experimental requirements.[10–12] As a result, these stimulator systems tend not to offer much flexibility in the range of parameters that are controlled by the investigators. Other systems such as Grass® stimulators[13,14] and the Ionoptix C-Pace®[15] have been developed to provide some parametric versatility.[16] However, these commercial

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products are designed for whole animal physiology (Grass) or standard two-dimensional (2D) tissue culture (C-Pace) and are of limited use for three-dimensional (3D) tissue engineering. Further, there is a cost barrier associated with these products that makes these units unattainable for many labs, especially those looking to do large-scale experimental deployments that would require correspondingly large numbers of stimulator systems. If new inexpensive, high-throughput options were available, rapid advances in understanding muscle development, disease, growth, and metabolism would be possible.

The aim of the current work was to develop a bioreactor system that could be used to stimulate muscle in either two or three dimensions *in vitro*. These bioreactors were designed around a reprogrammable custom electrical stimulation circuit that offers the flexibility of the commercial systems but with much lower cost of ownership. The system uses existing tissue culture plastic in conjunction with rapid prototyped enclosures to allow a single circuit to stimulate six samples simultaneously. Further, we show that the physiological stimulation parameters can directly affect the response to stimulation in 2D muscle cell culture and the function of 3D engineered muscles.

Materials and Methods

Stimulator circuit

The stimulator circuit is designed to provide programmed electrical pulses at amplitudes of up to 50 V and pulse widths down to 50 μs. Figure 1 shows the schematic for the circuit highlighting the three constituent parts, namely, the microcontroller, a DC–DC converter, and the pulse circuit.

The microcontroller used in this circuit was a Microchip 18LF4550, which is a low-power 16-bit chip with 35 IO pins, universal serial bus (USB) communications, and in-circuit serial programming (ICSP) programming capabilities. The LF version of the chip was chosen to allow the stimulator circuits to be used in a low-power mode such that they could run off 3-AA batteries instead of using power supplies. This feature may be of particular use in applications where *in vivo* stimulation is required. As shown in Figure 1, Pins B0 to B3 are connected as control output pins for the pulse circuit, and Pin B4 is used as a control pin for the DC–DC converter chip. Pins B5–B7 and master clear (MCLR) are connected to an RJ45 connector to allow for ICSP programming. To allow for future development and integration with sensors, Pins A0, A1, A4, and A5 are wired to a 10-pin connector along with

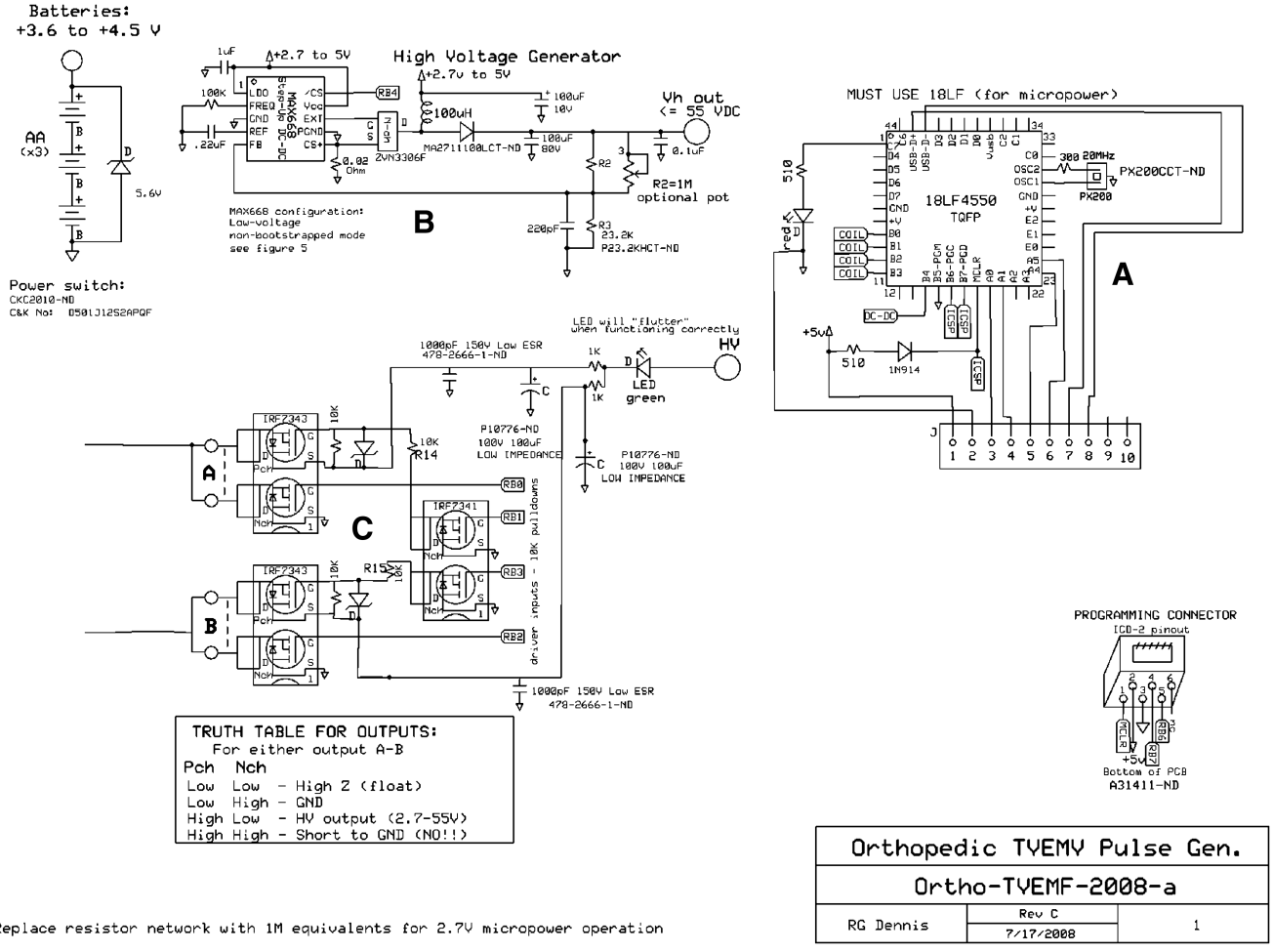

FIG. 1. Circuit diagram of the stimulation bioreactor. Schematic for the circuit highlighting (**A**) the microcontroller, (**B**) the DC–DC converter, and (**C**) the pulse circuit.

the USB data pins, +5 V, and ground. Pin C7 is connected through a 510 Ω resistor to a red LED to give a visual indication when the circuit is in operation.

The DC–DC converter circuit is based around the MAX668 step-up PWM controller (Maxim), which has an input voltage range of 1.8–28 V and provides up to 20 W output. The output voltage is set by the ratio of R2 and R3 according to the following equation 1:

$$V_{\text{out}} = \left(\frac{R2}{R3} + 1\right) \times V_{\text{ref}},$$

where V_{ref} is a reference voltage of 1.25 V supplied by pin 5 of the MAX668.

In the stimulator circuits, R2 is a potentiometer with a maximum resistance of 1 MΩ, R3 is a fixed resistor of 23.2 kΩ, giving a maximum V_{out} of 50 V. The voltage is generated through the high-frequency (500 kHz) switching of an inductor, and the design guidelines indicate the optimum value of this component can be calculated using the following formula (equation 2):

$$L_{\text{ideal}} = \frac{V_{\text{out}}}{(4 \times I_{\text{out}} \times f_{\text{osc}})}.$$

As the charging currents for the stimulation capacitors govern I_{out}, the maximum value is likely to be when both capacitors require to be charged from full discharge. In this case, the charging currents are limited by the series 1 kΩ resistors in each capacitor circuit, leading to a maximum value of 110 mA for the output current. Feeding this back into Equation 2, and using our chosen switching frequency of 500 kHz, this gives us an ideal inductance of 250 μH. As in previous studies, due to the relatively low duty cycle of the stimulations, lower inductances could be used where necessary; although values down to 22 μH were tried and found to work adequately, the optimum value chosen was 100 μH.

An input capacitor of 220 μF was used to reduce the current burden for the power supply, and a capacitor of similar value, but rated at greater than V_{out}, was used to reduce the voltage ripple and support the output during periods of high demand. All the components in the DC–DC converter circuit were chosen to minimize circuit losses that meant low DC resistance for the inductor and low equivalent series resistance for the capacitors. This criterion meant that the ZVN3306 field effect transistor was selected due to its low switching losses at the 500 kHz operating frequency. The high switching frequency also meant that a Schottky rectifier diode MA27111 (Panasonic) was used due to its high-speed response.

Pulse circuit

The pulse circuit used in the stimulators was based on a design used in previous *in vivo* systems.[17,18] Briefly, the circuit functions by charging capacitors to the required stimulation voltage. These are then discharged through muscle tissue, cell monolayers, or 3D muscle constructs via parallel metal electrodes using a growth medium as a conductive path. Using this method allows the quantification of the stimulation pulse using the rheobase (R50) and chronaxie (C50) definitions established elsewhere.[19] The capacitors are discharged through an arrangement of IRF7341 and IRF7343 FET transistors, which operate similar to an H-bridge motor drive system. The gates of the FETs are connected to the microcontroller such that it allows one electrode to be switched to ground immediately before connecting the other to the pulse capacitor for discharge. On completion of the stimulation, the FETs are switched off returning the electrodes to a high impedance state to decrease medium electrolysis. The stimulation pulses are designed to be bipolar to minimize electrolysis of the medium and oxidation of the electrodes, and these can either be alternating bipolar or paired bipolar. One of the modifications of this derivation to the original circuit is the use of individual pulse capacitors for each electrode. This modification was introduced as it was found that in the case of paired bipolar pulses, the second pulse was of lower amplitude than the first in high-frequency stimulation patterns. A second modification to the original was the introduction of a voltage divider circuit to the gates of the P-channel transistor in the IRF7343 FETs. This was due to the original configuration allowing the Vgs ratings for these transistors to be fatally exceeded at the higher stimulation amplitudes.

High-frequency limitations

The stimulator circuits are designed optimally for use at lower frequencies (up to 20 Hz); however, they can also be operated up to 100 Hz with some limitations. The main limiting factor in high-frequency operation is the need to recharge the capacitors in between stimulation pulses. To achieve this, we have modified some boards to include a parallel charging resistor of 100 Ω. This modification operates well at output voltages of up to 30 V for six wells and up to 40 V if three or fewer wells are used. Thus, the circuits can be used unaided in the high-frequency stimulation of 3D constructs, as the maximum amplitude used in these experiments is 20 V. For the 2D stimulation work, to mitigate some of the limitations at high frequency and voltage, the circuits can be used in conjunction with a standard amplifier such as the Crown K2 (Crown Audio, Elkhart, IN)

Bioreactor assemblies

Along with the stimulator circuits, the tissue culture plates are designed around standard tissue culture plastic. For 3D applications, the assembly comprises a baseplate that holds up to six 35 mm plates (Fig. 2A), whereas for 2D applications standard six-well plates are used (Fig. 2B). In both cases, four stimulating bioreactors can easily fit onto a single shelf in a standard cell culture incubator. The lid assembly is comprised of six sets of parallel electrodes embedded within a single lid. Figure 2 shows the prototype design that was used in the current study, whereas new rapid-prototyped lids are currently in use. The baseplates for the 3D assembly were designed using the Solidworks CAD package. Each baseplate has six wells in a 3×2 pattern, similar to standard six-well cell culture plates, and each well has a concentric cut-out in the bottom to allow imaging of the constructs during stimulation via an inverted microscope. The well centers and overall baseplate dimensions were chosen to mate with the electrode lids. The final designs were converted into STL file format and manufactured in polycarbonate using a StrataSys Titan FDM rapid prototyping system (StrataSys, Eden Prairie, MN).

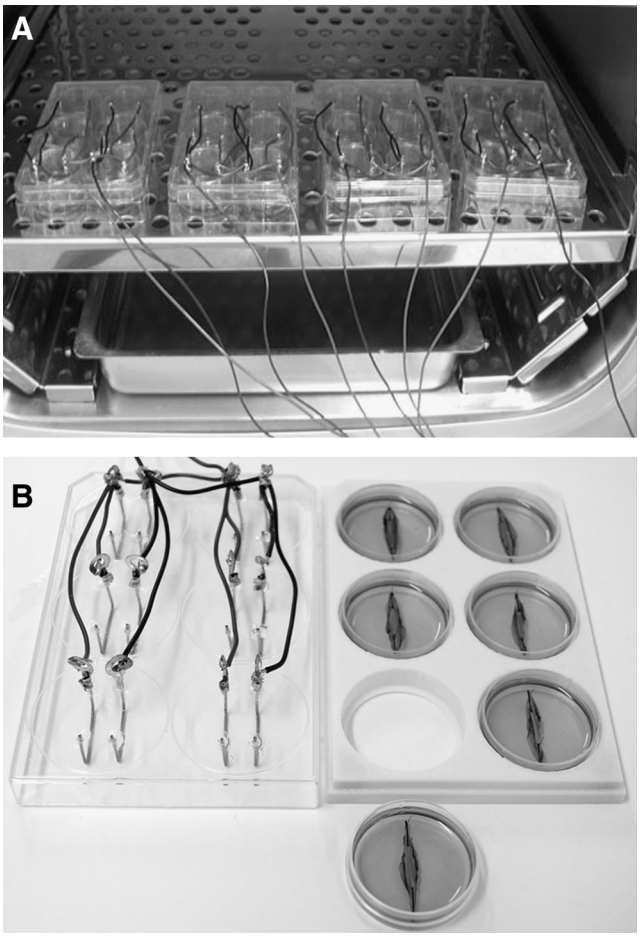

FIG. 2. Two-dimensional (2D) and three-dimensional (3D) stimulation bioreactor prototypes. Images of the prototype (**A**) 2D muscle-stimulating bioreactor and (**B**) 3D muscle-stimulating bioreactor.

The lid assemblies comprised the plastic lid into which parallel U-shaped stainless steel electrodes were embedded with 10 mm (for 3D applications) or 20 mm (for 2D applications) spacing. Crimp connectors on the end of the electrodes allowed for the soldering of wires to connect them to the stimulator circuit.

Circuit programming

Stimulation protocols were written in the C programming language and compiled using CCS PIC C compiler. The compiled hex code was then used to program the microcontrollers using Microchip MPLAB software and ICD-2 USB programmer. Within the program are two functions, STIM1 and STIM2, that send pins B0–B3 high or low in the correct sequence to activate the FETs and provide a pulse at one or other of the electrodes. Functions are also used to turn all the FETs off in between pulse sequences, thus ensuring the outputs are in the high-impedance state. This also provides a delay routine that can be looped, allowing long delay periods without the watchdog timer tripping. The main function initially switches on pin B4 to activate the DC–DC converter. This is followed by a delay period of 5 s to fully charge the capacitors before running the pulse sequences. Finally, the pulse sequences are activated with first STIM1 and then STIM2 functions being called, held for the required pulse width, and then switched off. The variable parameters in the program include the pulse width, pulse frequency, and the duty cycle. Thus, it is possible to produce more complex stimulation patterns with more physiologically relevant work to rest ratios.

Two-dimensional cell culture

C2C12 myoblasts were cultured in 35 mm plates (Dulbecco's modified Eagle's medium [DMEM], 10% fetal bovine serum, 1% Pen/strep; Invitrogen, Paisley, United Kingdom) until 90% confluent when they were differentiated (DMEM, 2% HS, 1% Pen/strep; Invitrogen). All experiments took place after 5 days of differentiation on fully formed myotubes. Immediately after stimulation, cells were collected in lysis buffer (50 mM Tris pH 7.5; 250 mM Sucrose; 1 mM ethylenediaminetetraacetic acid; 1 mM ethylene glycol tetraacetic acid [EGTA]; 1% Triton X-100; 1 mM NaVO4; 50 mM NaF; 0.10% dithiothreitol [DTT]; 0.50% programmable interface controller [PIC]), shaken at 4°C for 20 min (8,000 RPM), and centrifuged for 5 min at 12,000 RPM, and the supernatant was removed for protein determination. Protein concentration was determined using the DC protein assay (Bio Rad, Hertfordshire, United Kingdom) and equal aliquots of protein were boiled in Laemmli sample buffer.

Protein synthesis

Protein synthesis was assessed as the incorporation of [^{35}S] labeled methionine/cysteine (EasyTagTM Express Protein Labelling Mix, NEN Life Science Products). After stimulation, cells were maintained in serum-free DMEM. One hour before cell labeling, full DMEM was replaced with DMEM lacking methionine and cysteine. After this preincubation period, 3.66 μCi of a [^{35}S] methionine/cysteine mixture was added to each well and the cells were returned to the incubator for 2 h. Cells were lysed 3, 6, 12, and 24 h after stimulation in a buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM Igepal C630 (NP-40), 10% glycerol, 50 mM β-glycerophosphate, 50 mM NaF, and a protease inhibitor cocktail. Cell lysates were centrifuged for 2 min at 13,000 RPM and 15 μL of the supernatant was spotted on a Whatman paper in duplicate. Whatman papers were washed three times in 5% trichloroacetic acid containing cold methionine and cysteine, rinsed once in ethanol, and dried at 37°C. Four milliliters of scintillating liquid (Ultimagold; Perkin Elmer, Monza [Milano], Italy) was then added to the papers and [^{35}S] incorporation was counted in a scintillation counter (Beckman, High Wycombe, United Kingdom) for 2 min.

Three-dimensional cell culture

The 3D culture method was recently reported elsewhere.[20] Briefly, the muscle constructs were engineered between two 6-mm-long silk sutures set 12 mm apart on Sylgard poly dimethyl siloxane (PDMS)-coated dishes. Five hundred microliters of the growth medium containing 10 U/mL thrombin, 0.2 μg/mL genipin, and 50 ng/mL insulin-like growth factor-1 was added to the plate and agitated until it covered the entire surface; 200 μL of 200 mg/mL fibrinogen was added dropwise and the gels were left to polymerize for 1 h before addition of 100,000 cells. Cells were given fresh growth media every other day until the cells were ~90% confluent, at which point the constructs were switched to

DMEM supplemented with 10% horse serum and 1% penicillin/streptomycin for 2 days. After the second day in differentiation media, the constructs were moved to DMEM with 7% fetal bovine serum and 1% penicillin/streptomycin for the remainder of the experiment.

Construct testing

Functional testing of the C2C12 constructs was performed as described previously.[21] Briefly, one of the anchors was freed from the Sylgard substrate and attached to a custom-made force transducer via one the minutien pins. R50 and C50 were determined as described previously.[21] R50 was calculated as the electric field strength (V/mm) required to elicit 50% peak twitch force at a 4 ms pulse width. Chronaxie was calculated as the pulse width required to elicit 50% (peak twitch force) at twice R50. Maximum tetanic force was calculated at twice R50 at 4 ms using a 1 s train at 150 Hz for each individual construct. Cross-sectional area was calculated from the measured width of each construct (at its narrowest point), assuming a rectangular cross section and a depth of 500 μM. Specific force was calculated as kilonewtons per square meter: the force generated by the construct (kN) divided by its cross-sectional area (m^2).

Muscle cell stimulation

In 2D, the cells were stimulated at either 10 Hz (4×0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery) or 100 Hz (40×0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery). The 10 Hz protocol lasted 3 h, whereas the 100 Hz protocol lasted 30 min to model previous work performed using in tact muscle.[22] In 3D, the constructs were stimulated with 4×0.1 ms pulses delivered in a 400 ms train followed by 3.6 s recovery. This stimulation program was maintained throughout the entire 7 days of the study.

Statistical analysis

ANOVA (BrightStat.com) analysis followed by a Tukey HSD post-hoc test was used to determine differences between groups. Values are displayed as mean ± standard error of the mean, with statistical significance set at 0.05

Results

Two-dimensional electrical stimulation at different frequencies results in different cellular responses

To determine the effect of electrical stimulation on the cellular response of C2C12 muscle cells, 2D myotubes were stimulated with 0.3 ms pulses at low (10 Hz) and high (100 Hz) frequencies. The pulses were delivered in 400 ms trains with 3.6 s recovery. The length of the stimulation protocol was adopted from previous research *in vivo* that showed that 3 h at 10 Hz had no effect on muscle protein synthesis, whereas 30 min at 100 Hz increased protein synthesis.[22] Low-frequency electrical stimulation had no effect on the rate of muscle protein synthesis (control [CTL] 3 h = 4.66 ± 0.15; 10 Hz 3 h = 4.85 ± 0.13; CTL 12 h = 4.98 ± 0.16 10 Hz 12 h = 5.0 ± 0.07; CTL 24 h = 4.36 ± 0.05 10 Hz 24 h = 4.24 ± 0.09 fmol/min/mg) at any time point after stimulation (Fig. 3). In contrast, the higher frequency stimulation group showed a statistically significant increase in protein synthesis (3 h = 5.18 ± 0.10; 12 h = 5.50 ± 0.13; 24 h = 4.51 ± 0.09 fmol/min/mg).

Excitability of C2C12 muscle constructs

Since electrical damage is common when muscle is stimulated *in vitro* and C2C12 have been reported to have poor excitability, the C50 and R50 were determined for the fibrin-based C2C12 constructs. The excitability of control C2C12 muscle constructs was higher than has previously been reported for C2C12 constructs (R50 = 0.35 ± 0.025 V/mm; C50 = 474 ± 19.8 μs) 2 weeks after differentiation.[21] At 5 weeks after differentiation, the constructs were less excitable (R50 = 0.62 ± 0.018 V/mm; C50 = 896 ± 45.5 μs) and displayed a qualitative decrease in spontaneous contractile activity.

Three-dimensional stimulation increases muscle force production

To determine the effect of chronic electrical stimulation on the function of engineered C2C12 muscle constructs, R50 was first determined in 2-week-old constructs. The constructs were then stimulated at one, two, or four times R50

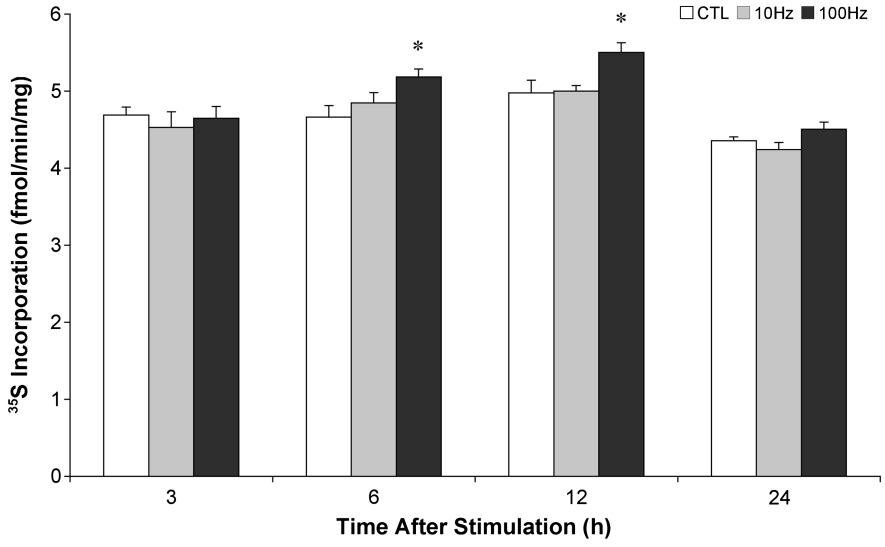

FIG. 3. Effect of stimulation at different frequencies on protein synthesis. C2C12 myotubes were stimulated with low-frequency (10 Hz) or high-frequency (100 Hz) electrical pulses, and the rate of protein synthesis 3, 6, 12, and 24 h later was determined. The pulses were delivered in 400 ms trains with 3.6 s recovery. The 10 Hz stimulation lasted for 3 h, whereas the 100 Hz stimulation lasted for 30 min. *Significantly higher than control ($p < 0.05$).

while maintaining the pulse width at 0.1 ms. The groups were stimulated with the 10 Hz protocol described above, where the muscles received one tetanus consisting of 4 pulses delivered in 400 ms with 3.6 s recovery.

Stimulation at 1.25 V/mm tended to increase force production and significantly decreased R50 (Fig. 4). At 2.5 V/mm both force and R50 were improved, whereas both force and R50 were negatively affected by stimulation at 5 V/mm. Chronaxie was unchanged in any of the groups These data indicate that, *in vitro*, high-voltage stimulation negatively affects 3D engineered muscle performance.

Discussion

We have developed a muscle electrical stimulation bioreactor that (1) can be used for either 2D or 3D muscle cultures, (2) is powered by batteries or inexpensive 5 V power sources, (3) is easily reprogrammed, and (4) allows investigator control over the voltage amplitude, stimulation frequency, pulse width, and work-to-rest ratio. Further, we have shown that important physiological differences result when changing not only the frequency, but also the amplitude of stimulation. Stimulating the 2D cultures at high frequencies resulted in an anabolic signal (increased protein synthesis), whereas stimulating 3D muscle constructs with high voltages decreased force generation and excitability.

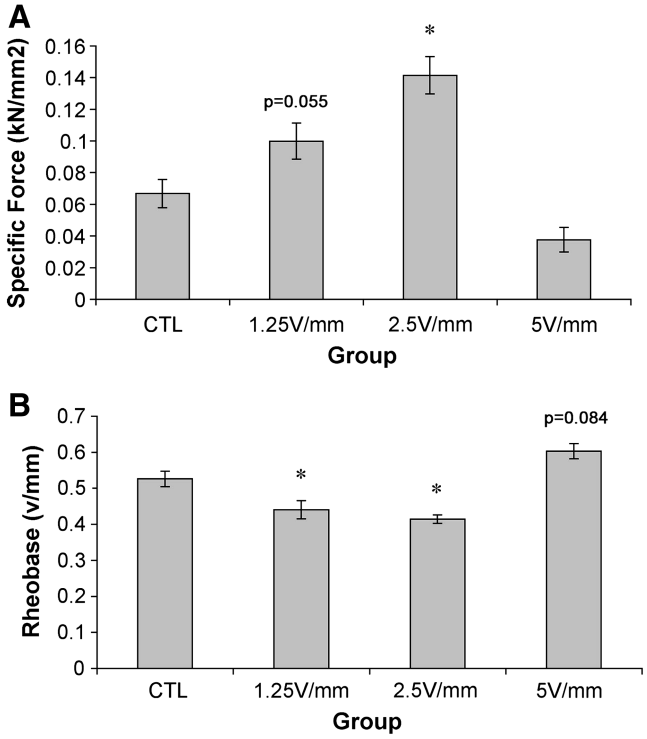

FIG. 4. Effect of 1-week stimulation with different pulse amplitudes and a pulse width of 0.1 ms on engineered muscle function. (**A**) Force and (**B**) rheobase (R50) (R50 determined with 4 ms pulse width) were determined in groups stimulated for 1 week at 1.25, 2.5, 5 V/mm, which corresponded to 1, 2, and 4 R50 at 0.1 ms. *Significantly different from control ($p < 0.05$). Note that decreased R50 is indicative of a more excitable muscle. Graph shows representative data ($n = 6$) of one of the two trails.

Although some commercial muscle stimulators are on the market, these units tend to be very expensive as they are engineered for a variety of functions, *in vivo* and *in vitro*, nerve and muscle, and so on, and as a result, many of the specific requirements for muscle tissue engineering applications are under represented. Further, although some of these systems effectively stimulate cells in 2D, none are currently designed for stimulating 3D muscle constructs. The stimulators reported here are highly flexible in their design parameters. Initially, these have been designed to use a six-well-plate format since this is routinely used within laboratories, allows moderate throughput, and is ideal for discovery-based bioreactors. As the ideal parameters for stimulation of the muscle cells in culture are determined, the system can be scaled as necessary for either clinical applications or delivery-based systems (that would increase the throughput of the system).

This is the first report of the C50 and R50 of fibrin-based engineered muscles. Previous reports on cocultures of C2C12 muscle cells and 10T1/2 fibroblasts[21] have indicated that the C2C12 cells had the excitability of chronically denervated skeletal muscle (R50 $\sim$1.93 V/mm, C50 $\sim$416 μs). Here we show that when the C2C12 constructs are formed using fibrin gel casting, the C50 is initially very similar (474 ± 19.8 μs), whereas the R50 value (0.35 ± 0.025 V/mm) is approximately fivefold lower. At 5 weeks in culture, the fibrin gel cast C2C12 constructs still have a lower R50, but C50 is greater (896 ± 45.5 μs) than the C2C12 and 10T1/2 cocultures. This suggests that higher amplitude pulses are required to excite skeletal muscle cells when surrounded by a dense extracellular matrix (the C2C12-10T1/2 cocultures), but smaller stimulus durations may be propagated efficiently. This might be a direct result of the electrical connections formed by fibroblasts within the culture[23]; once these cells within their dense extracellular matrix have been depolarized, they are able to readily propagate the electrical signal. However, since we have shown that greater pulse amplitudes result in a decrease in excitability over time, this suggests that the fibrin cast constructs may be better suited to long-term stimulation.

One of our initial hypotheses was that electrical stimulation would overcome the developmental block within the engineered muscles. Stimulation using 0.1 ms pulse widths and voltages of 1–2 times R50 improved force production and excitability, suggesting that using physiological models of electrical stimulation results in a more mature muscle. Even though we have not quantified the developmental state of the contractile machinery, excitability is an effective marker of the developmental state of muscle[24] and therefore can be used as a nondestructive biomarker of development.[7] *In vivo*, skeletal muscle contracts when an action potential is propagated along the surface of the muscle cell membrane causing depolarization of the t-tubular membrane and consequent calcium release from the sarcoplasmic reticulum. However, whether stimulation via parallel electrodes mimics this physiological depolarization has recently been questioned.[25] Unlike neural stimulation, Cairns *et al.*[25] demonstrated that using parallel electrodes and pulse widths longer than 0.25 ms results in direct calcium release from the sarcoplasmic reticulum and not surface membrane propagation using the t-tuble system. We therefore used 0.1 ms pulse widths to promote surface propagation of action potentials

in case this provides important developmental cues. However, it should be noted that we see the same increase in force and decrease in R50 using longer pulse widths (A. Khodabukus, unpublished observations).

The decrease in force and increase in R50 at 5 V/mm indicates that too much voltage has negative effects on engineered muscle performance. The most likely cause of this negative effect is electrochemical damage due to the high voltage.[26] Classically, electrochemical tissue damage is prevented by using stimulation protocols with the least energy, pure bipolar pulses, and high-impedance output when not stimulating.[26] The current data add to this previous work by suggesting that while the stimulus pulse energy is important, the voltage used plays a greater role in electrochemical damage than pulse width.

Electrical stimulation of muscle cells in 2D is becoming increasingly popular for studying the molecular effects of exercise on mitochondrial biogenesis, and insulin sensitivity. Our preliminary work showed that high-voltage stimulation (2–2.5 V/mm) or frequencies over 3 Hz would rapidly kill the 2D C2C12 muscle cells. Using the 3D C2C12 constructs to determine the R50 values allowed us to decrease the voltage used to 1 V/mm, and cell survival has not been a problem for up to 3 days when the cells pull off the plates (A. Philp, unpublished observations). Further, using the 3D C2C12 muscle constructs we noted that a fused tetanus occurs with stimulation frequencies in excess of 3 Hz (A. Khodabukus, unpublished observations). Accordingly, lower frequencies or different protocols were required. Therefore, when designing the 2D stimulation protocols a pulse train approach was developed. This means that for C2C12 muscle cells any frequency of stimulation over 1 Hz should be provided as a train of pulses followed by a rest period. Even when pulses are provided in this manner, varying the frequency of stimulation results in different physiological responses from the cells. At higher frequencies, there was a transient increase in the rate of protein synthesis. This is similar to what occurs after resistance exercise that results in muscle growth.[27] This difference has also been observed *in situ*. Using the same patterns of electrical stimulation, Atherton *et al*.[22] have shown that high-frequency stimulation results in an increase in whole muscle protein synthesis, whereas 10 Hz stimulation for 3 h does not. The ability to reproduce what has been observed in whole muscle and also maintain the muscle culture for at least 3 days makes this form of electrical stimulation a good model for *in vivo* muscle physiology.

Nedachi *et al*.[28] have previously performed a 24-hour, 1 Hz continuous stimulation protocol (IonOptix C-Dish®) on C2C12 cells in culture using a 2 ms pulse width and 0.67 V/mm. Although they did not determine the rate of protein synthesis, they did see an increase 5′adenosine monophosphate-dependent protein kinase (AMP kinase) activity. Since AMP kinase activity is increased by metabolic stress and can inhibit protein synthesis, it is possible that the low-frequency, long-duration stimulation that we performed resulted in greater AMP kinase activity and therefore less protein synthesis than the short-duration, high-frequency stimulation. Consistent with this hypothesis, Atherton *et al*.[22] have previously demonstrated that isometric contractions in isolated rat muscles with continuous low-frequency stimulation increased AMP kinase activity but did not increase protein synthesis, whereas 30 min of high-frequency stimulation did not alter AMP kinase phosphorylation but did increase protein synthesis. Interestingly, Nedachi *et al.* also reported an increase in slow myosin heavy chain and a reorganization of the sarcomeres that might translate into better force production.[28] This cellular reorganization might play a role in the increase in force production that we observe in 3D.

In conclusion, we have developed an inexpensive muscle electrical stimulation bioreactor for either 2D or 3D muscle cultures. This bioreactor allows researchers to apply physiologically relevant electrical stimulation patterns to tissue-engineered muscles and monolayers in culture. Further, we have shown that the voltage amplitude is a key determinant of the response of engineered muscle when stimulated *in vitro* and that stimulation frequency and duration are important in the anabolic response in 2D muscle cell culture. We believe that this bioreactor system will have wide application for both basic research, where it could be used to deduce how stimulation drives a muscle to be fast or slow, and clinical research, where it may provide insight into metabolic diseases such as diabetes.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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